US Environmental Protection Agency
Office of Pesticide Programs

Office of Pesticide Programs
Microbiology Laboratory
Environmental Science Center, Ft. Meade, MD

Standard Operating Procedure for
AOAC Use Dilution Method for Testing Disinfectants

SOP Number: MB-05-10

Date Revised: 12-04-12

SOP Number	MB-05-10
Title	AOAC Use Dilution Method for Testing Disinfectants
Scope	Describes the Use-dilution methodology (see 15.1) used to determine the efficacy of disinfectants against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Salmonella enterica* on hard surfaces.
Application	For product evaluations under the Antimicrobial Testing Program (ATP), a study protocol is developed which identifies the specific test conditions for a product sample such as contact time, dilutions, neutralizers, etc. Although the default growth medium specified in this SOP is synthetic broth, other growth media (e.g., nutrient broth) may be specified by the study sponsor.

	Approval Date
SOP Developer:	_____ Print Name: _____
SOP Reviewer	_____ Print Name: _____
Quality Assurance Unit	_____ Print Name: _____
Branch Chief	_____ Print Name: _____

Date SOP issued:	
Controlled copy number:	
Date SOP withdrawn:	

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1. Definitions	Abbreviations/definitions are provided in the text.
2. Health and Safety	Follow procedures specified in SOP MB-01, Laboratory Biosafety. The Study Director and/or lead analyst should consult the Material Safety Data Sheet for specific hazards associated with products.
3. Personnel Qualifications and Training	Refer to SOP ADM-04, OPP Microbiology Laboratory Training.
4. Instrument Calibration	Refer to SOP EQ-01, EQ-02, EQ-03, EQ-04 and EQ-05 for details on method and frequency of calibration.
5. Sample Handling and Storage	Refer to SOP MB-22, Disinfectant Sample Preparation, and SOP COC-01, Chain of Custody Procedures.
6. Quality Control	For quality control purposes, the required information is documented on the appropriate form(s) (see section 14).
7. Interferences	1. Any disruption of the *Pseudomonas aeruginosa* pellicle resulting in the dropping or breaking of the pellicle in culture before or during its removal renders that culture unusable in the use-dilution test. 2. Transferring the inoculated carriers into the disinfectant is a critical, technique-sensitive step. False positives can result from transfer of live organisms to sides of tubes due to contact or aerosol formation. 3. Viscous test chemicals may result in a substantial amount of product remaining on treated carriers following the contact time, which upon transfer to the primary subculture medium (neutralizer) produces cloudiness in the medium. This cloudiness may impact the recording of results.
8. Non-conforming Data	1. Sterility and/or viability controls do not yield expected results. 2. The mean log density for control carriers falls outside the specified range. Note: The prescribed minimum and maximum carrier counts also account for the addition of 5% organic soil to the inoculum. a. The mean *TestLD* for carriers inoculated with *S. aureus* and *P. aeruginosa* must be at least 6.0 (corresponding to a geometric mean density of 1.0×10^6) and not above 7.0 (corresponding to a geometric mean density of 1.0×10^7); a mean *TestLD* below 6.0 and above 7.0 invalidates the test, except for two retesting scenarios (outlined in the study protocol). b. The mean *TestLD* for carriers inoculated with *S. enterica* must be at least 5.0 (corresponding to a geometric mean density of 1.0×10^5)

	and not above 6.0 (corresponding to a geometric mean density of 1.0 × 10^6); a mean *TestLD* below 5.0 and above 6.0 invalidates the test, except for two retesting scenarios (outlined in the study protocol). 3. If contamination is present in the test system for more than one carrier for a passing product; no contamination is acceptable for a product test in which the product fails to meet the standard. 4. Management of non-conforming data will be specified in the study protocol; procedures will be consistent with SOP ADM-07, Non-Conformance Reports.
9. Data Management	Data will be archived consistent with SOP ADM-03, Records and Archives.
10. Cautions	1. There are time sensitive steps in this procedure including the use periods of the inoculated carriers and the test chemical. 2. Verify the volume of dilution blanks, neutralizer tubes, and subculture tubes in advance and adjust accordingly.
11. Special Apparatus and Materials	1. *Subculture media* (e.g., letheen broth, fluid thioglycollate medium). Note: Commercial media made to conform to the recipes provided in AOAC Methods 955.15, 964.02, and 955.14 may be substituted. 2. *Test organisms*. *Pseudomonas aeruginosa* (ATCC No. 15442), *Staphylococcus aureus* (ATCC No. 6538) and *Salmonella enterica* (ATCC No. 10708) obtained directly from ATCC. 3. *Culture media*. Note: Commercial media (e.g., synthetic broth) made to conform to the recipes provided in AOAC Methods 955.15, 964.02, and 955.14 may be substituted. a. *Synthetic broth*. Use for (10 mL) daily transfers and (10 mL) final test cultures. 4. *Trypticase soy agar (TSA)*. For use in propagation of the test organism to generate frozen cultures and as a plating medium for carrier enumeration. Alternately, TSA with 5% sheep blood (BAP) may be used. 5. *Sterile water*. Use reagent-grade water free of substances that interfere with analytical methods. Any method of preparation of reagent-grade water is acceptable provided that the requisite quality can be met. See Standard Methods for the Examination of Water and Wastewater and SOP QC-01, Quality Assurance of Purified Water for details on reagent-grade water. 6. *Carriers*. Polished stainless steel cylinders, 8 ± 1 mm outer diameter, 6 ± 1 mm inner diameter, 10 ± 1 mm length; type 304 stainless steel, SS 18-8

	(S & L Aerospace Metals, Maspeth, NY or Fisher Scientific catalog number 07-907-5Q as of July 2012). Use only carriers that passed bioscreening; refer to SOP MB-03, Screening of Stainless Steel Cylinders, Porcelain Cylinders and Glass Slide Carriers Used in Disinfectant Efficacy Testing. 7. *Specialized glassware.* For disinfectant, use autoclavable 25 × 100 mm tubes (Bellco Glass Inc., Vineland, NJ) for disinfectant. For glassware used to prepare test chemical, refer to SOP MB-22. 8. *Recirculating chiller unit*. For maintaining specified temperature of the test chemical. 9. *Transfer loops*. Make 4 mm inner diameter single loop at end of 50–75 mm (2–3 in.) Pt or Pt alloy wire No. 23 B&S gage or 4 mm loop fused on 75 mm (3 in.) shaft (available from Johnson Matthey, West Chester, PA 19380, USA). Fit other end in suitable holder. Bend loop at 30° angle with stem. 10. *Micropipettes*. For performing culture transfers and serial dilutions. 11. *Wire Hook*. For carrier transfer. Make 3 mm right angle bend at end of 50–75 mm nichrome wire No. 18 B&S gage. Place other end in suitable holder. 12. *Timer*. For managing timed activities, any certified timer that can display time in seconds. 13. *Sonicator* (ultrasonic cleaner). For conducting control carrier counts.
12. Procedure and Analysis	Prior to testing, perform the neutralization assay to determine if secondary subculture tubes are necessary (refer to SOP MB-17, Neutralization Confirmation). The AOAC Use-Dilution Test Processing Sheet (see section 14) must be used for tracking testing activities.
12.1 Test Culture Preparation	Refer to SOP MB-02 for the test microbe culture transfer notation. a. Defrost a single cryovial at room temperature and briefly vortex to mix. Add 10 µL of the thawed frozen stock (single use) to a tube containing 10 mL of growth medium (e.g., synthetic broth), vortex, and incubate at 36 ± 1°C for 24 ± 2 h. One daily transfer is required prior to the inoculation of a final test culture. Daily cultures may be

	subcultured for up to 5 days; each daily culture may be used to generate a test culture.[1] For *S. aureus* and *S. enterica* only, briefly vortex the 24 h cultures prior to transfer.[2] b. For the final subculture transfer, inoculate a sufficient number of 20 × 150 mm tubes containing 10 mL growth medium (e.g., synthetic broth) with 10 µL per tube of the 24 h culture; incubate 48-54 h at 36 ± 1°C. Do not shake the 48-54 h test culture. Record all culture transfers on the Organism Culture Tracking Form (see section 14).
12.2 Carrier Inoculation for *S. aureus*, *P. aeruginosa*, and *S. enterica*	Inoculate approximately 80 carriers; 60 carriers are required for testing, 6 for control carrier counts, and 1 for the viability control. a. For *P. aeruginosa*, remove the pellicle from the broth either by decanting the liquid aseptically into a sterile tube, by gently aspirating the broth away from the pellicle using a pipette, or by vacuum removal. Avoid harvesting pellicle from the bottom of the tube. Transfer test culture after pellicle removal into sterile 25 × 150 mm test tubes (up to approximately 20 mL per tube) and visually inspect for pellicle fragments. Presence of pellicle in the final culture makes it unusable for testing. Proceed as below in 12.2b. b. For *S. aureus* and *S. enterica*, using a vortex-style mixer, mix 48-54 h test cultures 3-4 s and let stand 10 min at room temperature before continuing. Remove the upper portion of each culture (e.g., upper ¾ or approximately 7.5 mL), leaving behind any debris or clumps, and pool culture into a sterile flask; swirl to mix. c. To achieve mean carrier counts within the appropriate range (see section 8), the final test culture may be diluted (e.g., one part culture plus one part sterile broth) prior to the addition of the OSL to the inoculum using the sterile culture medium used to generate the final test culture (e.g., synthetic broth). Use the diluted test culture for carrier inoculation within 30 min. Note: The use of a spectrophotometer to measure optical density (optical density at 650 nm) is recommended as a tool for assessing the need to dilute the final test culture. Always use sterile broth medium to calibrate the spectrophotometer. d. If organic burden is required for testing, the appropriate amount of organic burden is added to the pooled test culture prior to the inoculation of carriers. Swirl to mix. e. Drain the water from the carriers. Aseptically transfer 20 carriers

[1] Step not contained in the AOAC standard methods 955.14, 955.15, and 964.02.
[2] Step not contained in the AOAC standard methods 955.14 and 955.15.

	into each of the tubes containing the test culture. The test culture must completely cover the carriers; reposition carriers as necessary to ensure coverage. Alternatively, siphon off the water from the carriers and add 20 mL test culture directly to the carriers without transferring. f. Allow carriers to remain in inoculum for 15 ± 2 min. g. Drain the inoculum from the carriers with a pipette; avoid direct contact of the carrier with the tip of the pipette.[3] Briefly tap each carrier against the side of the tube to remove excess culture and place on end in vertical position in sterile Petri dish matted with 2 layers of Whatman No. 2 (or equivalent) sterile filter paper, making sure that carriers do not touch or fall over. Place no more than 12 carriers in a Petri dish. h. Dry carriers in incubator at 36 ± 1°C for 40 ± 2 min. Record the timed carrier inoculation activities on the AOAC Use-Dilution Test Processing Sheet (see section 14). Perform efficacy testing within two hours of drying.
12.3 Enumeration of viable bacteria from carriers (control carrier counts)	a. Assay dried carriers in 2 sets of three carriers, one set immediately prior to conducting the efficacy test and one set immediately following the test. Select one carrier from each of 6 Petri dishes. b. Place each inoculated dried carrier into a tube containing 10 mL of letheen broth and sonicate in an ultrasonic cleaner for 1 min ± 5 s. Record the time of sonication on the AOAC Use-Dilution Test Processing Sheet (see section 14). c. For sonication, place tubes into an appropriately sized glass beaker with tap water to the level of the letheen broth in the tubes. Place the beaker in an ultrasonic cleaner so that the water level in the beaker is even with the water level fill-line on the tank. Fill the tank with tap water to the water level fill-line. Hold the beaker so that it does not touch the bottom of the tank and all 3 liquid levels (inside the test tubes, inside the beaker, and inside the tank) are approximately the same. d. After sonication, briefly mix and make serial ten-fold dilutions in 9 mL dilution blanks of PBDW. Refer to the AOAC Use-Dilution Test Carrier Counts Form (see section 14). If the serial dilutions are not made and plated immediately, keep the sonicated tubes at 2-5°C until this step can be done. Complete the dilutions and plating within 2 h

[3] Step not contained in the AOAC standard methods 955.14, 955.15, and 964.02.

	after sonication. Alternatively, pool the letheen broth from the tubes with the carriers after sonication and brief vortexing for each set of three carriers. Serially dilute and plate an aliquot of the pooled media (30 mL). The average carrier count per set will be calculated. e. Briefly vortex each serial dilution tube prior to plating. Plate 0.1 mL aliquots of appropriate dilutions in duplicate on TSA or BAP using spread plating. Plate appropriate dilutions to achieve colony counts in the range of 30-300 colony forming units (CFU) per plate. Spread inoculum evenly over the surface of the agar. Plates must be dry prior to incubation. f. Incubate plates (inverted) at 36 ± 1°C for up to 48 ± 2 h. g. Count colonies. Plates that have colony counts over 300 will be reported as TNTC. Record counts on the AOAC Use-Dilution Test Carrier Counts Form (see section 14). See section 13 for data analysis.
12.4 Disinfectant Sample Preparation	a. Prepare disinfectant sample per SOP MB-22. b. Equilibrate the water bath and allow it to come to 20 ± 1°C or the temperature specified (±1°C). Prepare the disinfectant dilutions within 3 hours of performing the assay unless test parameters specify otherwise. Record the time of disinfectant preparation on the AOAC Use-Dilution Test Processing Sheet (see section 14). c. Dispense 10 mL aliquots of the disinfectant into 25 × 100 mm test tubes, one tube per carrier. Place tubes in the equilibrated water bath for approximately 10 min to allow disinfectant to come to specified temperature. Record the temperature of the water bath and recirculating chiller before and after testing on the AOAC Use-Dilution Test Information Sheet (see section 14).
12.5 Test Procedure	a. Sequentially transfer the carriers from the Petri dish to the test tubes containing the disinfectant at appropriate intervals (e.g., 30 second intervals). b. Add one carrier per tube and swirl the tube using 2-3 gentle rotations before placing it back in the water bath. Avoid intense swirling and agitation of the carrier. For a contact time of 10 min, the carrier must be deposited in the tube within ± 5 s of the prescribed drop time. c. Using alternating hooks, flame-sterilize the hook and allow it to cool after each carrier transfer. When lowering the carriers into the disinfectant tubes, neither the carrier itself nor the tip of the wire hook can touch the interior sides of the tube. If the interior sides of

	the tube are touched, repeat the carrier. d. Following the exposure time, sequentially transfer the carriers into subculture/neutralizer media. Remove the carrier from the disinfectant with a sterile hook, tap it against the interior sides of the tube to remove the excess disinfectant, and transfer it into the subculture tube within ±5 s. Avoid tapping the carrier against the upper third of the tube. Avoid contact of the carrier to the interior sides of the subculture tube during transfer. e. Recap the subculture tube and shake thoroughly. Incubate at 36 ± 1°C for 48 ± 2 h. f. If a secondary subculture tube is deemed necessary to achieve neutralization, then transfer the carrier from the primary tube to a secondary tube of sterile medium after a minimum of 30 ± 5 min at room temperature from the end of the initial transfer. Within 25-60 min of the initial transfer, transfer the carriers using a sterile wire hook to a second subculture tube. Move the carriers in order but the movements do not have to be timed. Thoroughly shake the subculture tubes after all of the carriers have been transferred. Incubate both the primary and secondary subculture tubes 48 ± 2 h at 36 ± 1°C. Record the results for both tubes (a carrier set) after this time. g. Record timed events on the AOAC Use-Dilution Test Time Recording Sheet for Carrier Transfers (see section 14).
12.6 Sterility and viability controls	a. Viability controls. Place 1 (or 2) dried inoculated untreated carrier(s) into separate tubes of the neutralizing subculture broth (if primary and secondary media are different). Incubate tubes with the efficacy test. Report results as + (growth) or 0 (no growth) as determined by presence or absence of turbidity. Growth should occur in both tubes. Record results on AOAC Use-Dilution Test Results Sheet (see section 14). b. Sterility controls. Place one sterile, uninoculated carrier into a tube of neutralizing subculture broth. Incubate tube with the efficacy test. Report results as + (growth), or 0 (no growth) as determined by presence or absence of turbidity. Growth should not occur in the tube. Record results on AOAC Use-Dilution Test Results Sheet (see section 14).
12.7 Results	a. Gently shake each tube prior to recording results. Record results as + (growth) or 0 (no growth) as determined by presence or absence of turbidity, on the AOAC Use-Dilution Test Results Sheet (see section

	14). b. If secondary subculture tubes are used, the primary and secondary subculture tubes for each carrier represent a "carrier set." A positive result in either the primary or secondary subculture tube is considered a positive result for a carrier set. c. Specialized neutralizer/subculture medium such as Dey/Engley broth will not show turbidity; rather the presence of pellicle at the surface of the medium (for *P. aeruginosa*) or a color change to the medium (yellow for growth of *S. aureus* or *S. enterica*) must be used to assess the results as a positive or negative outcome. i. Use viability controls for comparative determination of a positive tube. ii. If the product passes the performance standard, a minimum of 20% of the remaining negative tubes will be assayed for the presence of the test microbe using isolations streaks on TSA or BAP. Record preliminary results and conduct isolation streaks at 48 ± 2 h, however, continue to incubate negative tubes for up to an additional 24 hours to confirm the results.[4]
12.8 Confirmatory Steps for Test Microbes	a. Confirm a minimum of three positive carrier sets per test. If there are less than three positive carriers, then confirm each carrier. If secondary subculture tubes are used and both tubes are positive in a carrier set, select only the tube with the carrier for confirmatory testing. b. For a test with greater than 20 positive carrier sets, confirm at least 20% by Gram staining, and a minimum of 4 positive carrier sets by Gram staining, solid media, and appropriate biochemical and antigenic analyses to ensure the identity of the organism. c. See Attachment 1 for Gram stain reactions, cell morphology, and colony characteristics on solid media. d. For additional confirmation steps refer to the appropriate Confirmation Flow Chart for *S. aureus, P. aeruginosa,* and *S. enterica* (see Attachment 3). e. If confirmatory testing determines that the identity of the unknown was not the test organism, annotate the positive entry (+) on the results sheet to indicate a contaminant was present.
12.9 Re-use of	a. After use, autoclave all carriers. Carriers for which test results were

[4] Step not contained in the AOAC standard methods 955.14, 955.15, and 964.02.

Stainless Steel Carriers	negative may be reused after cleaning. Carriers that are positive are re-cleaned and screened biologically (see SOP MB-03, Screening Carriers) before re-use. These carriers may be reused if the biological screening test results in no growth. The extra inoculated carriers, positive control,[5] and those used for carrier counts may be autoclaved, re-cleaned, and used again.
13. Data Analysis/ Calculations	Calculations will be computed using a Microsoft Excel spreadsheet (see section 14). Both electronic and hard copies of the spreadsheet will be retained. Counts up to 300 and their associated dilutions will be included in the calculations.
14. Forms and Data Sheets	1. Attachment 1: Typical Growth Characteristics of strains of *P. aeruginosa*, *S. aureus*, and *S. enterica* 2. Attachment 2: Culture Initiation Flow Chart for *S. aureus*, *P. aeruginosa*, and *S. enterica* 3. Attachment 3: Confirmation Flow Charts for *S. aureus*, *P. aeruginosa* and *S. enterica* 4. Test Sheets. Test sheets are stored separately from the SOP under the following file names: Organism Culture Tracking Form — MB-05-10_F1.docx Test Microbe Confirmation Sheet (Quality Control) — MB-05-10_F2.docx AOAC Use-Dilution Test Time Recording Sheet for Carrier Transfers — MB-05-10_F3.docx AOAC Use-Dilution Test Information Sheet — MB-05-10_F4.docx AOAC Use-Dilution Test Results Sheet (1°) — MB-05-10_F5.docx AOAC Use-Dilution Test Results Sheet (1°/2°) — MB-05-10_F6.docx Test Microbe Confirmation Sheet — MB-05-10_F7.docx AOAC Use-Dilution Test Carrier Counts Form — MB-05-10_F8.docx AOAC Use-Dilution Test Processing Sheet — MB-05-10_F9.docx Carrier Count Spreadsheet MS Excel spreadsheet: Carrier Count Template_UDT_v3 — MB-05-10_F10.xlsx AOAC Use-Dilution Test Carrier Counts Form (Pooled Carriers) — MB-05-10_F11.docx

[5] Step not contained in the AOAC standard methods 955.14, 955.15, and 964.02.

15. References	1. Official Methods of Analysis. July 2012. 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, (Methods 955.14, 955.15, and 964.02). 2. Krieg, Noel R. and Holt, John G. 1984. Bergey's Manual of Systematic Bacteriology Volume 1. Williams & Wilkins, Baltimore, MD. *P. aeruginosa* p. 164, *S. enterica* p. 447. 3. Sneath, P., Mair, N., Sharpe, M.E., and Holt, J. eds. 1986. Bergey's Manual of Systematic Bacteriology Volume 2. Williams & Wilkins, Baltimore, MD. *S. aureus* p. 1015. 4. Package Insert – Gram Stain Kit and Reagents. Becton, Dickinson and Company. Part no. 882020191JAA. Revision 07/2011. 5. Package Insert – Catalase Reagent Droppers. Becton, Dickinson and Company. Part no. L001237. Revision 06/2010. 6. Package Insert – Staphaurex Plus. Remel. Part no. R30950102. Revised 11/23/07. 7. Package Insert – Oxidase Reagent Droppers. Becton, Dickinson and Company. Part no. L001133. Revision 06/2010. 8. Package Insert – Wellcolex Colour Salmonella. Remel. Part no. R30858301. Revised 10/17/07.

Attachment 1

Typical Growth Characteristics of strains of *P. aeruginosa*, *S. aureus*, and *S. enterica* (see ref. 15.2 and 15.3).

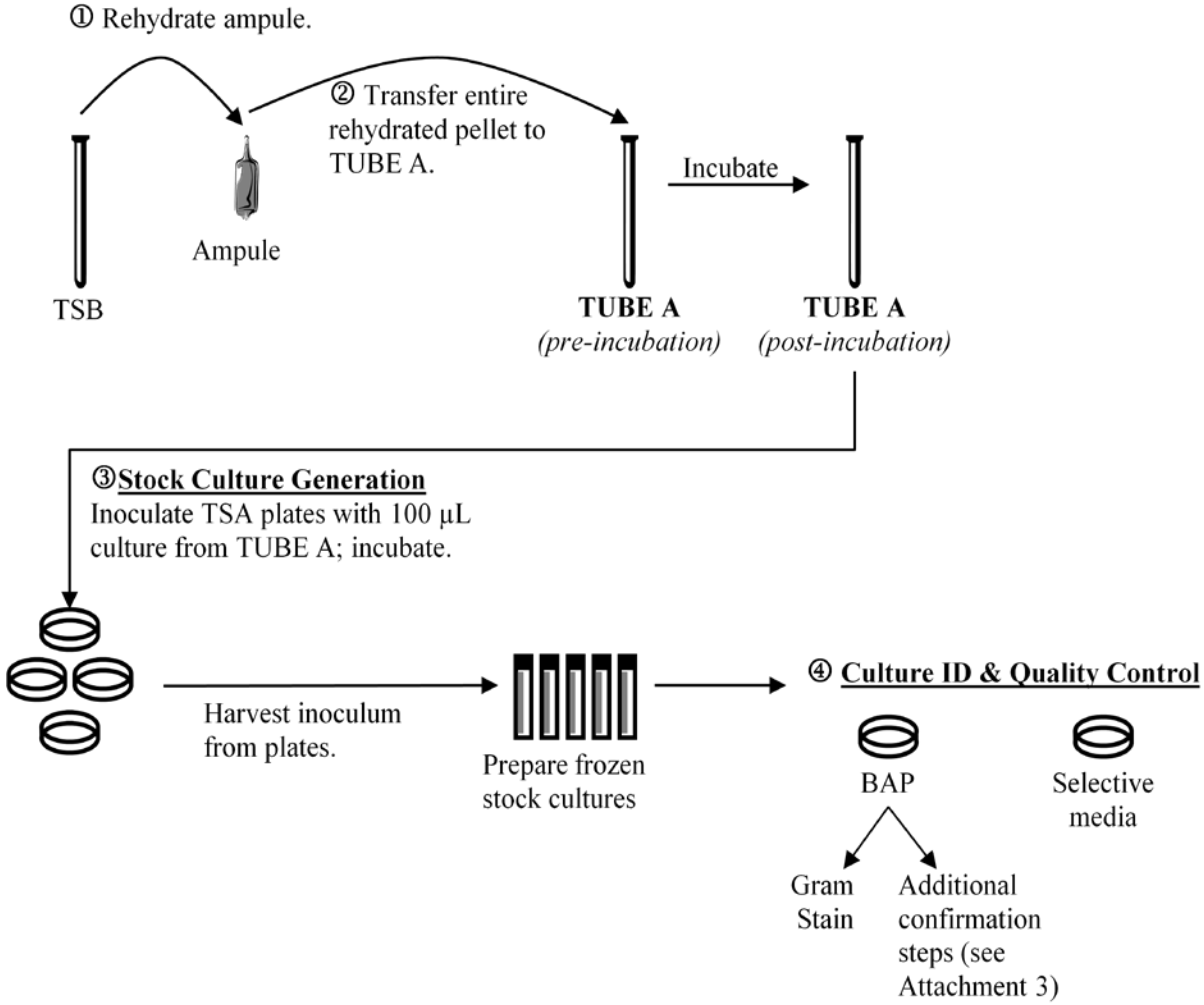
	*P. aeruginosa**	*S. aureus**	*S. enterica**
Gram stain reaction	(-)	(+)	(-)
Typical Growth Characteristics on Solid Media			
Mannitol Salt	No Growth	circular, small, yellow colonies, agar turning fluorescent yellow	N/A
Cetrimide	circular, small, initially opaque, turning fluorescent green over time; agar fluorescent yellowish green	No Growth	N/A
Xylose lysine deoxycholate (XLD) agar	N/A	N/A	Round, clear red colonies with black centers
Blood agar (BAP)	flat, opaque to off-white, round spreading (1), metallic sheen, slightly beta hemolytic	small, circular, yellow or white, glistening, beta hemolytic	entire, glistening, circular, smooth, translucent, low convex, non-hemolytic
Typical Microscopic Characteristics			
Cell dimensions	0.5-1.0 µm in diameter by 1.5-5.0 µm in length*	0.5-1.5 µm in diameter*	0.7-1.5 µm in diameter by 2.0-5.0 µm in length*
Cell appearance	straight or slightly curved rods, single polar flagella, rods formed in chains	spherical, occurring singly, in pairs and tetrads, sometimes forming irregular clusters	straight rods, peritrichous flagella

*After 24±2 hours

(1) Test organism may display three colony types: a) circular, undulate edge, convex, rough and opaque; b) circular, entire edge, convex, smooth and translucent; c) irregular, undulate edge, convex, rough, spreading, and translucent. Pyocyanin is not produced.

Attachment 2

Culture Initiation and Stock Culture Generation Flow Chart for *S. aureus*, *P. aeruginosa*, and *S. enterica*



A1. Preparation of Frozen Stock Cultures. Refer to SOP MB-02 for establishment of the organism control number.

a. Initiate new stock cultures from lyophilized cultures of *Pseudomonas aeruginosa* (ATCC 15442), *Staphylococcus aureus* (ATCC 6538), and *Salmonella enterica* (ATCC 10708) from ATCC within 18 months.

b. Open ampule of freeze dried organism as indicated by ATCC. Using a tube containing 5-6 mL of TSB, aseptically withdraw 0.5 to 1.0 mL and rehydrate the lyophilized culture. Aseptically transfer the entire rehydrated pellet back into the original tube of broth designated as "TUBE A". Mix well.

c. Incubate broth culture (TUBE A) at 36 ± 1ºC for 24 ± 2 hours. Record all manipulations on the Organism Culture Tracking Form (see section 14).

d. Using a sterile spreader, inoculate a sufficient number of TSA plates (e.g., 5 to 10 plates per organism) with 100 µL each of the culture. Incubate plates at 36 ± 1°C for 24 ± 2 h.

e. Following incubation, add 5 mL cryoprotectant solution (TSB with 15% v/v glycerol) to the surface of each agar plate. Re-suspend the cells in this solution using a sterile spreader or a sterile swab and aspirate the cell suspension from the surface of the agar. Transfer the suspension into a sterile vessel. Repeat by adding another 5 mL of cryoprotectant to the agar plates, re-suspend the cells, aspirate the suspension and pool with the initial cell suspension.

f. Mix the pooled contents of the vessel thoroughly. Immediately after mixing, dispense approximately 1.0 mL aliquots into cryovials (e.g., 1.5 mL cyrovials). Perform QC of stock cultures concurrently with freezing (see section A2: QC of Stock Cultures).

g. Place and store the cryovials at -70°C or below; these are the frozen stock cultures. Stock cultures may be used up to 18 months; reinitiate using a new lyophilized culture.[6] These cultures are single-use only.

A2. QC of Stock Cultures.

a. Conduct QC of the pooled culture concurrently with freezing. Streak a loopful on a plate of BAP. In addition, for *S. aureus* and *P. aeruginosa*, streak a loopful onto both selective media (MSA and Cetrimide); for *S. enterica*, streak a loopful onto XLD. Incubate all plates at 36 ± 1ºC for 24 ± 2 hours.

b. Following the incubation period, record the colony morphology as observed on the BAPs and selective media plates (including the absence of growth) and Gram stain. See Attachment 1 for details on cell and colony morphology, colony characteristics on selective media, and stain reactions.

c. For each organism, perform a Gram stain (refer to 15.5) from growth taken from the BAPs according to the manufacturer's instructions. Observe the Gram reaction by using brightfield microscopy at 1000X magnification (oil immersion).

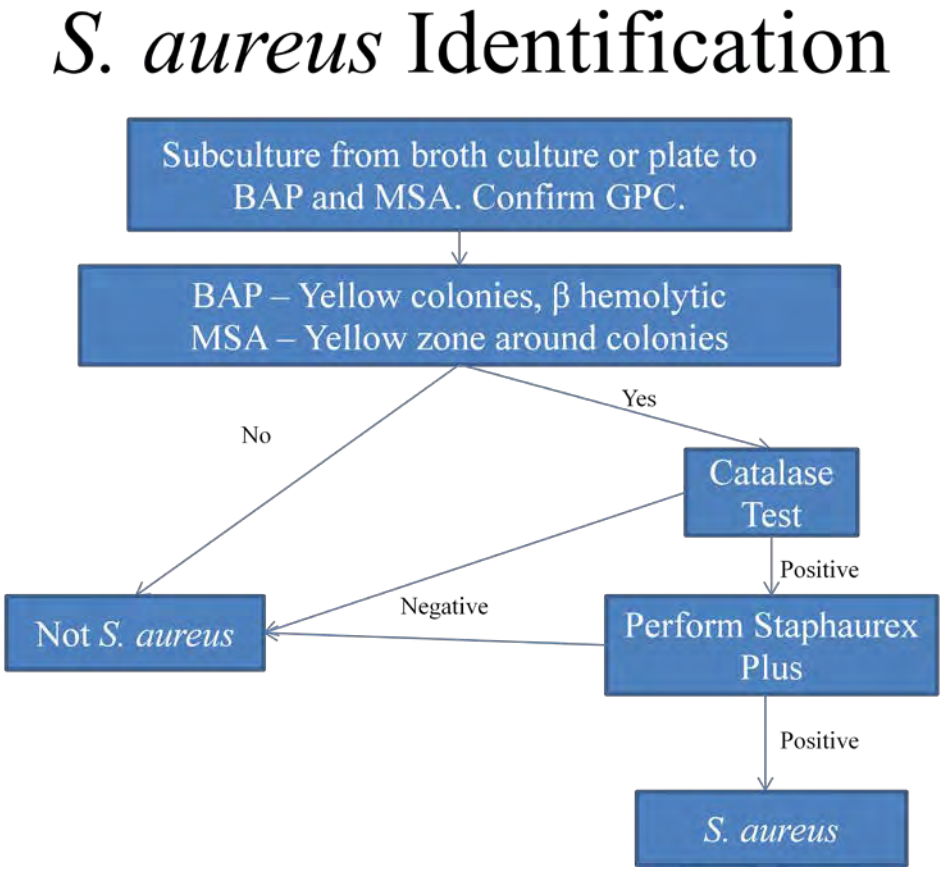
d. For additional confirmation steps refer to the appropriate Confirmation Flow Chart for *S. aureus*, *P. aeruginosa*, and *S. enterica* (see Attachment 3). Refer to 15.6-15.9 for instructions.

e. Record all confirmation results on the Test Microbe Confirmation Sheet (Quality Control) (see section 14).

[6] Step not contained in the AOAC standard methods 955.14, 955.15, and 964.02.

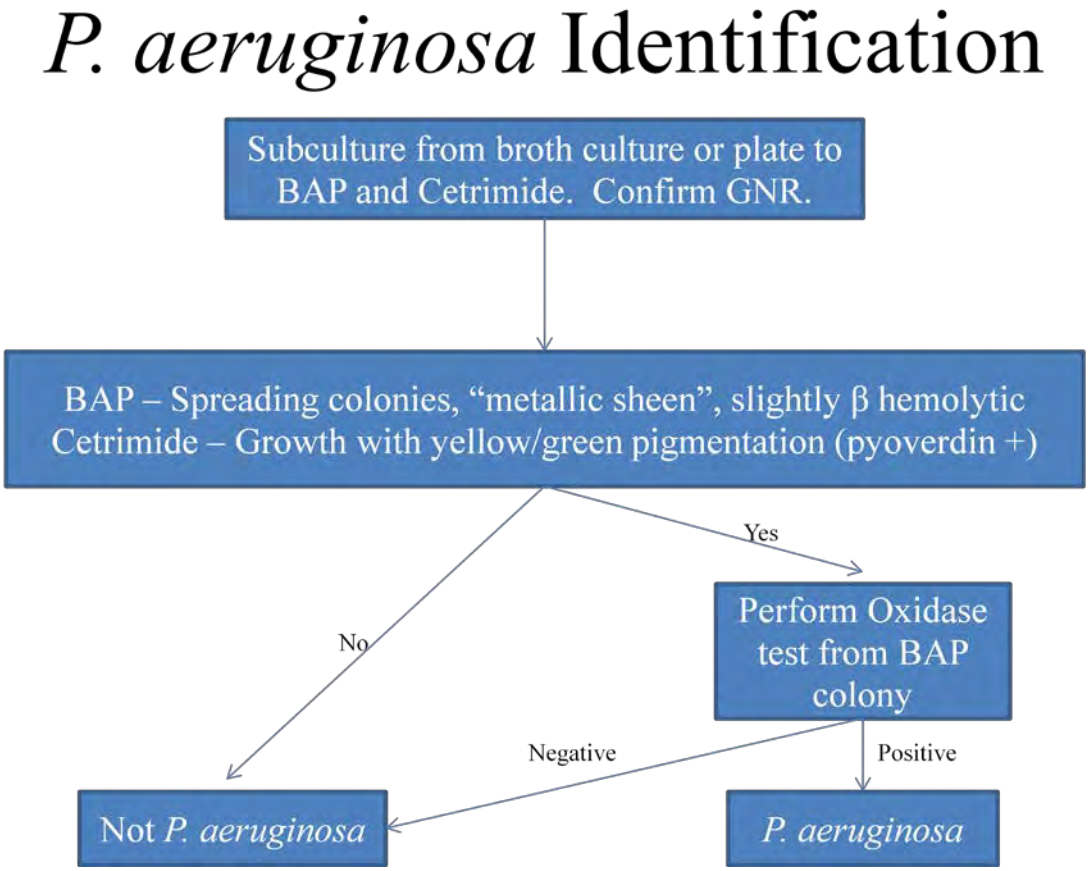
Attachment 3

Confirmation Flow Chart for *S. aureus*



Attachment 3 (cont.)

Confirmation Flow Chart for *P. aeruginosa*



Attachment 3 (cont.)

Confirmation Flow Chart for *S. enterica*